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USE OF FOLLICLE STIMULATING HORMONE FOR REDUCTION OF SPERMATOZOA CHROMOSOMAL
ABERRATION IN MALES

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USE OF FOLLICLE STIMULATING HORMONE FOR REDUCTION OF SPERMATOZOA CHROMOSOMAL ABERRATION IN MALES

5 **Field of the invention**

This invention relates to the use of a substance selected from Follicle Stimulating Hormone (FSH) and FSH variants for reducing gamete chromosomal alterations in the male. More specifically, the invention provides the use of a substance selected from FSH and FSH variants in men suffering from spermatozoa aneuploidy, notably diploidy
10 and disomy. The invention further comprises the use of pharmaceutical formulations containing Follicle Stimulating Hormone (FSH) and FSH variants for the preparation of a pharmaceutical composition for the treatment and/or prevention of gamete chromosomal alterations in the male.

15 **Background of the invention**

A considerable percentage of infertile men have an abnormal karyotype and, therefore, these subjects produce gametes with chromosomal alterations. Furthermore, having a normal karyotype does not exclude the possibility that spermatozoa with chromosomal alterations are present, since errors in chromosomal segregation can occur during the
20 mitotic and/or meiotic division in spermatogenesis. Based on the first data available in the literature (*Vegetti et al., 2000 Human Reproduction, 15(2), 351-365*), it has been suggested that approximately 0.3–1.08% of the spermatozoa of normal men have numerical chromosomal aberrations and that this percentage becomes higher when examining the spermatozoa of men with oligospermia (0.7–9.44%) and teratospermia
25 (1.3–3.9%). These alterations appear to be related to the sex chromosomes and autosomes, particularly chromosomes 1, 18, and 21.

Numerical chromosomal aberration characterized by extra or missing chromosomes is called “aneuploidy”.

30 Different types of aneuploidies are known and they are designated according to the kind of chromosomal numerical aberration: for example, the presence of one additional chromosome compared to the normal number in diploid cells is known as trisomy ($2n + 1$); the absence of one chromosome in a homologous pair in diploid cells is known as

monosomy ($2n-1$), while the loss of an entire chromosomal pair in diploid cells is known as nullisomy ($2n-2$), wherein n is the number of types of chromosomes.

By analogy, in the case of haploid cells, such as gametes, the term "aneuploid" characterizes gametes having a chromosomal numerical aberration, such as for example having an extra chromosome or missing one chromosome. These "aneuploid" gametes will form, when fused with a normal gamete, a zygote having an abnormal number of chromosomes (aneuploidy). Most of the time, the aneuploid zygotes die during the time between conception and birth. However, in some cases, the zygote becomes an offspring affected by "aneuploidy" and the consequences on the carrier of this chromosomal numerical aberration depends on the chromosomes which are involved.

The development of gametic aneuploidy of autosomes and sex chromosomes results from errors, such as failed separation of brother chromatids that occur during mitosis of spermatogonia and during the first and second meiotic division or such as failed separation of homologous chromosomes in meiosis. When the chromosomes fail to separate during the first meiotic phase, all the produced spermatozoa will have an aberrant number of chromosomes (only $2n$:diploids or $0n$). If the chromosomes fail to separate during the second meiotic phase, only 2 haploid cells out of the 4 coming from the same spermatocyte will be affected by chromosomal numerical aberration.

Therefore production of gamete aneuploidies constitutes a serious genetic risk factor as they induce aneuploidy in the offsprings which causes a lot of abnormalities in the fetus and in the viable infants.

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For example, aneuploidy in the infant can cause infant later infertility, miscarriages and perinatal mortality of the infant, congenital malformations, mental retardation, and abnormal behavior (Hook, 1985, in: *Aneuploidy: Etiology and mechanisms*. (eds) V.L. Dellarco, P.E. Voytek, A. Hollander, pp. 7-33. New York: Plenum; Hecht et al., 1987, in: *Aneuploidy. Part A: Incidence and etiology*. (eds) B.K. Vig, A.A. Sandberg, 9-49. New York: Alan R. Liss), increased sensitivity to infectious diseases, high propensity of leukaemia or early development of Alzheimer disease in the offspring.

Aneuploidies in autosomes such as monosomies are always lethal for the foetus whereas trisomies are non lethal only when they involve chromosomes 13, 18 and 21 but are dramatically handicapping for the offspring.

5

Examples of common sex chromosome aneuploidy are found in patients with Klinefelter Syndrome (47, XXY) which is the most common form of aneuploidy in men and in patients (47, XYY) which is another form of aneuploidy discovered by Sandberg and co-workers.

10

The presence of spermatozoa chromosomal abnormalities (both numerical and structural) has been noted in a considerable percentage of infertile patients (*Bourrouillou et al., 1985, Hum Genet, 71, 366-367*) and it is widely accepted that using assisted fertilization techniques allows these patients to procreate, increasing the risk of

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having children with chromosomal alterations.

Aneuploid gametes also develop in subjects with oligoasthenoteratozoospermia (OAT) with normal chromosomal sets since noxious testicular pathogens can disrupt the delicate process of chromosomal segregation during spermatogenesis. For this reason,

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evaluating the frequency of aneuploidy in spermatozoa is becoming a fundamental stage in the diagnostic course of infertile patients, particularly if they intend to undergo assisted fertilization techniques.

25

Published data indicate that patients with OAT have an increased percentage of spermatozoa with hereditary chromosomal aneuploidy. This increased frequency of aneuploidy reduces the fertilizing capability of spermatozoa during assisted fertilization techniques (*Storeng et al., 1998, Acta Obstet Gynecol Scand, 77(2), 191-197*). Considering the fact that spermatozoa separation methods used during fertilization *in vitro* and embryo transfer (FIVET) do not modify the percentage of aneuploid sperm,

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patients with OAT are at great risk of producing children with chromosomal aneuploidy (*Pfeffer et al., 1999, Fertil Steril, 72, 472-478*).

Research in this area has become more clinically relevant in the past few years with the development of intra-cytoplasmic sperm injection (ICSI). ICSI has been shown to be extremely useful for the treatment of infertility, however transmission of cytogenic defects to offspring is a major concern with this fertilization technique and has been
 5 observed (*Ushijima et al., 2000, Human Reproduction, 15(5), 1107-1111*).

For this reason, spermatogenic aneuploidy should be assessed in all patients who intend to undergo assisted fertilization techniques.

It would be desirable to develop methods for reducing the rate of aneuploidy such as
 10 diploidy in spermatozoa, especially in men who intend to undergo Assisted Reproduction Techniques.

Follicle-stimulating hormone (FSH) is known for its role in the initial development of Sertoli cells and in their stimulation for controlling spermatogonia. It is suggested by
 15 experimental studies that FSH is present in Sertoli cells and in round germinal cells that both express the FSH receptor (*Baccetti et al., 1998, Human Reproduction, 12, 9, 1955-1968*).

Furthermore, exogenous FSH therapies induced improvements in the structural
 20 characteristics of spermatozoa visualized through electron microscopy (*Baccetti et al., 1997, The FASEB Journal, 12, 1045-1054*).

FSH is used to induce spermatogenesis in men suffering from oligospermia. A regimen using 150 IU FSH, 3 times weekly in combination with 2'500 IU hCG (human
 25 Chorionic Gonadotrophin) twice weekly has been successful in achieving an improvement in sperm count in men suffering from hypogonadotrophic hypogonadism (*Burgues et al., 1997, Hum. Reprod., 12, 980-6*). High dose FSH (150 IU) has been used to treat idiopathic oligospermia (*Iacono et al., 1996, J. Urol., 102, 81-4*).

30 Summary of the invention

It is an object of the invention to provide a method for the reduction and/or prevention of gamete chromosomal alterations in a male, notably spermatozoa aneuploidy,

including diploidy and disomy, preferably in men intending to undergo assisted fertilization techniques, whereby a pharmaceutically active amount of FSH or FSH variant is administered to the male in need thereof.

5 In a first aspect, the invention provides a use of a substance selected from Follicle Stimulating Hormone (FSH) and FSH variants for the preparation of a pharmaceutical composition for the treatment and/or the reduction of gamete chromosomal alterations in a male, notably spermatozoa aneuploidy, including diploidy and disomy.

10 In a second aspect, the invention provides a method for reducing gamete numerical chromosomal alterations in a male subject, notably spermatozoa aneuploidy, including diploidy and disomy, comprising administering an effective dose of a substance selected from FSH and a FSH variant to the patient.

15 In a third aspect, the invention provides a method for preventing the occurrence of chromosomal aberrations in the offspring of a male subject, comprising administering an effective dose of a substance selected from FSH and a FSH variant to the male subject prior to conception.

20 **Detailed description of the invention**

The following paragraphs provide definitions of various terms, and are intended to apply uniformly throughout the specification and claims unless an otherwise expressly set out definition provides a different definition.

25 "Oligozoospermia" is characterized by a semen containing a low sperm count. It is usually diagnosed in men when sperm concentration $<20 \times 10^6/\text{ml}$.

"Asthenozoospermia" is characterized by a semen having an impaired sperm motility. It is usually diagnosed in men when fewer than 50% spermatozoa have forward
30 progression or fewer than 25% spermatozoa have rapid linear progression.

"Teratozoospermia" by a semen having sperm with an abnormal morphology. It is usually diagnosed in men when fewer than 30% spermatozoa have normal morphology.

"Oligoasthenoteratozoospermia" is characterized by a semen having all the three previously cited variables for sperm quality, which are disturbed. According to WHO criteria, it is diagnosed when patients display the following parameters: density $<20 \times 10^6$ spermatozoa/mL, motility of grade 3 $<25\%$ and/or motility of grade 2 plus 3 $<50\%$, and normal morphology in less than 30% of spermatozoa.

Exemplary diseases where chromosomal aberration are involved include Down's syndrome, Klinefelter's syndrome (XXY), Turner's syndrome (XO), Triplo-X syndrome, Tetra-X syndrome, Penta-X syndrome, XYY syndrome, monosomies or polysomies of any chromosome including 7, 10, 11, 13, 18, 21, X and Y, sex chromosome aneuploidy in men with chromosomal mosaicism (XY/XXY) and multiple X chromosomes (e.g. XXXXY; XXXYY).

The term "administer" or "administering" means to introduce a formulation of the present invention into the body of a patient in need thereof to treat a disease or condition.

The term "chromosomal aberration" is used for chromosomal abnormalities and includes chromosomal numerical aberrations such as polyploidy and aneuploidy which includes the presence of at least an extra chromosome or the absence of one chromosome. Aneuploidy in haploid cells such as gametes includes diploidy.

~~"Aneuploidy" and "polyploidy" are conditions in which a cell has a number of~~
chromosomes different from the usual haploid number ("n") or diploid number ("2n").
In humans, the normal haploid number (number of chromosome in haploid cells such as gametes) is 23, and the diploid number is 46. A normal somatic cell should have a
diploid chromosome content of 23 pairs, or 46 chromosomes in total.

An aneuploid somatic cell has a number of chromosomes that is other than twice the normal haploid number ($2n$). For example, an aneuploid somatic cell can be a cell having a trisomy, i.e., a cell having three copies of one chromosome, or a monosomy, i.e., a cell having a single copy of one chromosome. Aneuploidy can result from chromosomal non-disjunction during mitosis. A polyploid cell is a cell having a number of chromosomes that is some multiple of the normal haploid number greater than the usual diploid number, i.e., $2n$. For example, a polyploid cell can be triploid ($n=69$) or tetraploid ($n=92$).

10 In a haploid cell, such as a gamete, the natural state is haploid, meaning n chromosomes. Aneuploidy means that the cell has a chromosome number that is other than n . For example, one chromosome may be missing ($n-1$), or an extra chromosome may be added ($n+1$) such as in disomy, or the number of chromosomes can be doubled such as in diploidy ($2n$).

15

The term "patient" means a male mammal that is treated for a disease or condition. Patients are of, but not limited to, the following origin, human, ovine, porcine, equine, bovine, rabbit and the like.

20 The term "Assisted Reproduction Technology" includes IVF (In Vitro Fertilization), ICSI (intra-cytoplasmic sperm injection).

By "effective amount", is meant an amount of FSH sufficient to reduce gamete chromosomal alterations in a male subject, especially to reduce the rate of aneuploidy, including diploidy and disomy in a male patient. The amount of FSH can be routinely determined by those of skill in the art. The amount of the compound actually administered will typically be determined by a physician, in the light of the relevant circumstances, including the condition to be treated, the chosen route of administration, the actual compound administered, the age, weight, and response of the individual patient, the patient's endogenous FSH levels, and the like. The "effective amount" can alternatively be achieved by the duration of the treatment. Typically, the duration of the

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treatment includes treatments of at least one month, three month, six months or one year.

The expression "pharmaceutically acceptable" is meant to encompass any carrier or salt
 5 which does not substantially interfere with the effectiveness of the biological activity of the active ingredient and that is not toxic to the host to which is administered.

The term "recombinant" refers to preparations of FSH or FSH variants that are produced through the use of recombinant DNA technology.

10

The rate of aneuploidy, diploidy or disomy can be determined through the evaluation of numerical chromosomal abnormalities in ejaculated spermatozoa for example by fluorescence *in situ* hybridisation (FISH), especially multicolour FISH. FISH involves hybridisation of specific DNA probes labelled with fluorochromes to complementary
 15 DNA sequences on target chromosomes, followed by detection of the bound probes under a fluorescence microscope as described in *Shi et al., 2001, Reproduction, 121, 655-666*. To perform FISH analysis on semen samples from donors with extremely low quantities of sperm, a microwave decondensation/co-denaturation technique has been developed to increase analyzable sperm numbers for use in fluorescence *in situ*
 20 hybridization (FISH) (*Rademaker et al., 2001, Cytogenet Cell Genet. 95(3-4), 143-5*). Example of a FISH protocol for determining the rate of aneuploidy or diploidy is detailed in Example n° 1.

The levels of basal serum testosterone can be measured by commercially available
 25 ELISA/chemiluminescent immunoassays.

The expression "FSH variant" is meant to encompass those molecules differing in amino acid sequence, glycosylation pattern or in inter-subunit linkage from human FSH but exhibiting FSH-activity. Examples include CTP-FSH, a long-acting modified
 30 recombinant FSH, consisting of the wild type α -subunit and a hybrid β -subunit in which the carboxy terminal peptide of hCG has been fused to the C-terminal of the β -subunit of FSH, as described in (*La Polt et al., 1992, Endocrinology, 131, 2514-2520* or *Klein et*

al., 2003, *Human Reprod.* 2003, 18, 50-56). Also included is single chain CTP-FSH, a single chain molecule, consisting of the following sequences (from N-terminal to C-terminal):

β FSH	β hCG-CTP(113-145)	α FSH
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- 5 wherein β FSH signifies the β -subunit of FSH, β hCG CTP (113-145) signifies the carboxy terminal peptide of hCG and α FSH signifies the α -subunit of FSH, as described by Klein et al., 2003. Other examples of FSH variants include FSH molecules having additional glycosylation sites incorporated in the α - and/or β -subunit, as disclosed in WO 01/58493, particularly as disclosed in claims 10 and 11 and FSH
10 molecules with inter-subunit S-S bonds, as disclosed in WO 98/58957.

The FSH variants referred to herein also include the carboxy terminal deletions of the beta subunit that are shorter than the full length mature protein.

- 15 The FSH used in the invention can be "recombinant", from a natural source or obtained by chemical synthesis.

The FSH variants in the invention can be "recombinant" or obtained by chemical synthesis.

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An example of recombinant DNA technology for producing FSH is described in WO 85/01958.

- 25 One example of a method of expressing FSH using recombinant technology is by transfection of eukaryotic cells with the DNA sequences encoding an alpha and beta subunit of FSH, whether provided on one vector or on two vectors with each subunit having a separate promoter, as described in European patent nos. EP0211894 and EP0487512.

- 30 Another example of the use of recombinant technology to produce FSH is by the use of homologous recombination to insert a heterologous regulatory segment in operative

connection to endogenous sequences encoding one or both of the subunits of FSH, as described in European patent no. EP0505500.

Also contemplated are methods such as those disclosed in WO99/57263, wherein one of the subunits is inserted heterologously into a cell, and the other subunit is expressed by
5 activation of genomic sequences by insertion of a heterologous regulatory segment by homologous recombination. The method of the invention may be used with any of these methods of expressing FSH.

Recombinant human FSH that is suitable for use in the invention is, for example,
10 GONAL-fTM (FSH, Follitropin alpha) from SERONO.

The FSH used in accordance with the present invention may be purified from biological sources, such as from urinary sources, e.g. urinary FSH or urofollitropin (uFSH). Acceptable methodologies include those described in *Hakola, 1997, Molecular and*
15 *Cellular Endocrinology, 127:59-69; Keene et al., 1989, J. Biol. Chem., 264:4769-4775; Cerpa-Poljak et al., 1993, Endocrinology, 132:351-356; Dias et al. 1994, J. Biol. Chem., 269:25289-25294; Flack et al., 1994, J. Biol. Chem., 269:14015-14020 and Valove, et al., 1994, Endocrinology, 135:2657-2661; U.S. Patent 3,119,740 and US Patent no. 5,767,067.*

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FSH or a FSH variant may be formulated for injection, either intra-muscular or subcutaneous, preferably subcutaneous.

The FSH formulation may be freeze-dried, in which case it is dissolved in water for
25 injection just prior to injection. The FSH formulation may also be a liquid formulation, in which case it can be injected directly, without prior dissolution.

The FSH formulation may be in both single dose or multiple dose liquid formats, in vials, or ampoules. Single dose formats must remain stable and potent in storage prior
30 to use. Multi-dose formats must not only remain stable and potent in storage prior to use, but must also remain stable, potent and relatively free of bacteria over the multiple use regimen administration period, after the seal of the ampoule has been compromised.

For this reason, multi-dose formats often contain a bacteriostatic agent, such as, for example, benzyl alcohol, meta-cresol, thymol or phenol, preferably benzyl alcohol or meta-cresol. Single dose formulations may also comprise a bacteriostatic agent.

- 5 FSH or FSH variants may be formulated with known excipients and stabilizers, for example, sucrose and mannitol. The formulation may also comprise an antioxidant, such as methionine. It may further comprise a surfactant, such as TWEEN (preferably TWEEN 20) or Pluronic (preferably Pluronic F68).
- 10 FSH is currently formulated for intra-muscular (IM) or subcutaneous (SC) injection. It is supplied in a lyophilised (solid) form in vials or ampoules of 75 IU/vial and 150 IU/vial with a shelf life of one and a half to two years when stored at 2-25°C. A solution for injection is formed by reconstituting the lyophilised product with water for injection (WFI). Depending on the patient's response, up to three cycles of treatment
- 15 with increasing doses of FSH can be used. With lyophilised formulations, the patient is required to reconstitute a new vial of lyophilised material with diluent and administer it immediately after reconstitution on a daily basis: for example: [Package insert N1700101A, published in February 1996, for Fertinex™ (purified urofollitropin for injection) for subcutaneous injection, by Serono Laboratories, Inc., Randolph, MA] and
- 20 Puregon® (Follitropin beta for injection) for subcutaneous injection, by N.V.Organon, BH OSS, Netherlands).

Examples of formulations for FSH are listed below:

- EP0618808 (Applied Research Systems ARS Holding N.V.) discloses a pharmaceutical
- 25 composition comprising a solid intimate mixture of gonadotrophin and a stabilising amount of sucrose alone or in combination with glycine.

- EP0448146 (AKZO N.V.) discloses a stabilized gonadotrophin containing lyophilisate comprising one part by weight of a gonadotrophin; and 200 to 10,000 parts by weight of
- 30 a dicarboxylic acid salt stabilizer associated with the gonadotrophin.

EP0853945 (Akzo Nobel N.V.) discloses a liquid gonadotrophin-containing formulation characterised in that the formulation comprises a gonadotrophin, stabilising amounts of a polycarboxylic acid or a salt thereof and of a thioether compound.

- 5 WO00/04913 (Eli Lilly and Co.) discloses a formulation comprising FSH or a FSH variant, containing an alpha and beta subunit, and a preservative selected from the group consisting of phenol, m-cresol, p-cresol, o-cresol, chlorocresol, benzyl alcohol, alkylparaben (methyl, ethyl, propyl, butyl and the like), benzalkonium chloride, benzethonium chloride, sodium dehydroacetate and thimerosal, or mixtures thereof in
10 an aqueous diluent.

The levels of FSH may be increased in a male suffering from gamete chromosomal alterations, by raising endogenous FSH levels through the administration of FSH agonists, for example the FSH agonists described in WO 0209706 and WO 0008015.

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In one embodiment, the invention provides a use a substance selected from Follicle Stimulating Hormone (FSH) and FSH variants for the preparation of a pharmaceutical composition for the treatment and/or the reduction of gamete chromosomal alterations in a male, notably numerical aberrations in spermatozoa such as gamete aneuploidy,
20 including diploidy and disomy.

In another embodiment, the invention provides a method for treating and/or preventing a disease or disorder associated with gamete chromosomal alterations in a male, notably numerical aberrations in spermatozoa such as gamete aneuploidy, including diploidy
25 and disomy, comprising administering to a male subject in need thereof an effective amount of a substance selected from FSH and FSH variants, wherein the subject can be human or animal.

In another embodiment, the invention provides a method for reducing gamete
30 chromosomal alterations in a male, notably numerical aberrations in spermatozoa such as gamete aneuploidy, including diploidy and disomy, comprising administering an effective dose of a substance selected from FSH and FSH variants to the patient.

In another embodiment, the invention provides a method for preventing the occurrence of chromosomal aberrations in the offspring of a male subject, comprising administering an effective dose of a substance selected from FSH and FSH variants to the male, prior to conception.

In another embodiment, the invention provides a method of *in vitro* fertilization, including ISCI, comprising the step of treating the donor of spermatozoa, prior collection of spermatozoa, with an amount of FSH or FSH variant sufficient to prevent or reduce chromosomal aberrations in the spermatozoa.

In a preferred embodiment of the invention, the male is human.

In another preferred embodiment of the invention, the substance is FSH.

In another preferred embodiment of the invention, the substance is rFSH.

In another preferred embodiment of the invention, the male presents a numerical chromosomal aberration in spermatozoa.

In another preferred embodiment of the invention, the numerical chromosomal aberration in spermatozoa is diploidy.

In another preferred embodiment of the invention, the numerical chromosomal aberration in spermatozoa is sex chromosome disomy.

In another preferred embodiment of the invention, the male presents an aneuploidy in spermatozoa at least or about 1%.

In another preferred embodiment of the invention, the male presents a diploidy in spermatozoa of at least or about 0.5%.

Another preferred group of patient is represented by males presenting a diploidy of at least or about 0.8%.

Another preferred group of patient is represented by males presenting an aneuploidy and
5 a basal serum level of total testosterone of at least or about 5,000 pg/ml.

In another preferred embodiment of the invention, the administration of the substance is performed on alternate days.

10 In another preferred embodiment of the invention, the substance is administered at or about 75 to 300 IU/dose, preferably at 150 IU/dose.

In another preferred embodiment of the invention, the patient will undergo Assisted Reproduction Technology such as IVF (*in vitro* fertilization) and ISCI (intracytoplasmic
15 sperm injection).

In another preferred embodiment of the invention, the treatment of the male patient with a substance selected from FSH and FSH variants, is performed during at least a time selected from one week, one month, three months, six months and one year, preferably
20 at least three months before undergoing Assisted Reproduction Technology.

In one more preferred embodiment, the invention provides a use of rFSH for the preparation of a pharmaceutical composition for the treatment and/or the reduction of spermatozoa aneuploidy in a patient.
25

~~In another more preferred embodiment, the invention provides for reducing~~
spermatozoa aneuploidy in a patient, comprising administering an effective dose of a substance selected from rFSH to the patient.

30 The invention will now be described by means of the following Examples, which should not be construed as, in any way, limiting the present invention. The Examples will refer to the Figures specified here below.

Description of the figures:

Figure 1 represents the variation of the percentages of total aneuploidy at the different stages of the treatment, i.e. 150 UI/day on alternate days (-45: 45 days before the treatment; 0: the day of the treatment before the treatment; 90: 90 days after the beginning of the treatment).

Figure 2 represents the variation of the percentages of diploidy respectively at the different stages of the treatment, i.e. 150 UI/day on alternate days (-45: 45 days before the treatment; 0: the day of the treatment before the treatment; 90: 90 days after the beginning of the treatment).

Figure 3 represents the variation of the percentage of total disomy at the different stages of the treatment, i.e. 150 UI/day on alternate days (-45: 45 days before the treatment; 0: the day of the treatment before the treatment; 90: 90 days after the beginning of the treatment).

Abbreviations:

CEP (Chromosome Enumeration Probes); **DHEA** (Dehydroepiandrosterone); **DTT** (dithiothreitol, Biorad); **EDTA** (ethylenediaminetetraacetic acid); **FIVET** (Fertilization *In Vitro* and Embryo Transfer); **FISH** (Fluorescent IN SITU Hybridization); **FSH** (Follicle Stimulating Hormone); **hCG** (human Chorionic Gonadotropin); **ICSI** (Intracytoplasmic Sperm Injection); **IM** (intra-muscular); **IVF** (*In Vitro* Fertilization); **IU** (International Unit); **LIS** (3,5 di-iodosalicylic acid lithium salt); **OAT** (oligoasthenoteratozoospermia); **rFSH** (Recombinant FSH); **RT** (room temperature); **SC** (subcutaneous); **TSH** (Thyroid Stimulating Hormone); **uFSH** (urinary FSH); **USP** (United States Pharmacopeia); **WFI** (Water For Injection).

EXAMPLES

The invention will be illustrated by means of the following examples which are not to be construed as limiting the scope of the invention.

EXAMPLE 1: Effect of GONAL-f™ (recombinant hFSH from SERONO) on aneuploidy in spermatozoa of human infertile males (8 patients).

a) Selection of infertile patients

Infertility of patients is determined after an andrological visit comprising spermiogram
5 record, hormonal measurement (Androstenedione (A), total testosterone (Ttot), DHEAs, Prolactine (PRL), TSH, free tyroxine (FT4)) and microdeletion of chromosome Y.

Normal values are the following: A: 1200-5000 ng/ml, T tot: 1500-11400 pg/ml, Prl: 2-15 ng/ml; FT4: 50-120 ng/ml; FSH: 0.7 – 10 mIU/ml.

Blood samples are obtained between 9-11 a.m. Measurements are performed by
10 commercial RIA or ELISA kits.

b) Aneuploidy/diploidy rates in sex chromosomes determination by FISH analysis before treatment

Fresh sperm samples were washed with 150 mM NaCl and 10 mM Tris-HCl (pH 8),
15 smeared on glass slides and dried in air. They were then fixed in 3:1 methanol-acetic acid for 10 min. The slides were dehydrated in 70%, 80% and 100% cold ethanol and air dried. Samples were swollen treated with 0.01 M DTT (dithiothreitol, Biorad) in 0.1M Tris-HCl (pH 8) and then 20 mM LIS (3,5 di-iodosalicylic acid lithium salt, Sigma) in the same buffer, checking sperm head swelling. The slides, rinsed in 2X SSC
20 (pH 7) and air dried, were dehydrated and denatured in 70% formamide (Aldrich) 2X SSC at 73° for 4 min. They were then quickly dehydrated in a graded ethanol series at 0°C and air dried.

During this last step, CEP (Chromosome Enumeration Probes, Vysis, IL, USA) - satellite DNA probes for chromosomes X, Y and 18 directly labelled with different
25 fluorochromes were used. The probe mix was denatured for 5 min at 73°C in a water bath. Hybridization was carried out at 37°C in a moist chamber for 12 hours. The slides were then washed with 0.4X SSC-0.3% NP40 for 2 min at 73°C, quickly in 2X SSC-0.1% NP40 at RT and finally mounted with DAPI 125 ng/ml in antifade solution (Vysis, IL, USA). A total of 3500-5000 spermatozoa were analyzed by triple color
30 FISH.

SCORING CRITERIA

The overall hybridization efficiency was >99%. Sperm nuclei were scored according to criteria of *Martin et al. 1995, Mol. Reprod. Dev., 42(1): 89-93*. Sperm nuclei are scored only if they are intact, non-overlapping and have a clearly defined border.

In the case of aneuploidy, the presence of sperm tail was confirmed. A sperm was considered disomic if the two fluorescent spots are of same colour, comparable in size, shape and intensity and positioned inside the edge of the sperm head at least one domains apart.

Diploidy was recognized by the presence of two double fluorescent spots, following the above criteria. Observation and scoring was performed on a Leitz Aristoplan Optic Microscope equipped with fluorescence apparatus, with a triple band-pass filter for Aqua, Orange, Green Fluorocromes (Vysis, IL, USA) and a monochrome filter for DAPI.

Patients having the following characteristics are selected:

Diploidy represented the 50.24% of total aneuploidy observed before treatment.

-Positive test for the presence of diploidy $\geq 0.8\%$ by FISH measurement on ejaculated spermatozoa as described above.

-FSH serum levels < 10 UI/ml .

Patients presenting at least one of these characteristics are excluded from the study:

-Body mass index [(BMI = weight (kg)/height (m²) x 100] <30;

-Presence of infection in the spermatid passageways (i.e. chlamydia, ureaplasma, mycoplasma) at baseline;

-Known autoimmune disorders;

-Thyroid disorders and/or pathologies;

-Chronic hepatopathies;

-Presence of concomitant pathologic conditions that are contraindicated in FSH treatment.

c) Treatment protocol:

Patients are administered with a dose equal to 150 UI (2 vials s.c.) on alternate days for 3 months.

5 d) Aneuploidy/diploidy rates determination by FISH analysis after treatment

The rates of aneuploidy and diploidy in sex chromosomes are measured by FISH analysis after treatment following the protocol detailed above (b).

10 A reduction in diploidy by -54% was found on a sample size of 8 consecutive patients (95% confidence interval: -20% to -87%; n=3; p=0.02).

A reduction in the frequency of sex chromosome aneuploidy was found on a sample size of 8 consecutive patients.

15

EXAMPLE 2: Effect of GONAL-fTM on diploidy and aneuploidy in 19 human infertile males.

a) Patient selection:

20 A total of 23 infertile male patients from age 18 to 55 are selected. Their infertility is determined according to the protocol detailed in Example 1.

b) Treatment:

19 patients are administered with a dose equal to 150 UI (2 vials s.c.) on alternate days for 3 months and 4 patients are not treated.

25 The percentages of total aneuploidy, diploidy and total disomy is measured by the FISH test (see Example 1) 45 days before the treatment (-45), on the day of treatment before the treatment (0).

~~Total aneuploidy is the calculated as the sum of diploidy and total disomy.~~ The percentages of specific disomies (sex chromosomes disomy and chromosome 18 disomy) are calculated. Sex chromosome disomy is further analysed into detailed percentages of disomy for chromosomes XX, YY and XY.

30

c) End-point measurements:

After 3 months of treatment (90), the patients undergo the same examinations that are performed at the beginning of the study and the FISH tests are repeated to measure each aneuploidy, diploidy and disomy parameters.

5 d) Statistical analysis of the measurements:

The statistical significances of differences between averages are calculated using the protocol detailed in Example 1.

10 The effects of the 3-month-treatment with GONAL-f™ on total aneuploidy and diploidy are reported respectively on Figures 1 and 2.

A reduction of 38.4% in total aneuploidy and a reduction of 40.7% in diploidy are observed.

15 The effect of the 3-month-treatment with GONAL-f™ on total disomy is reported in Figure 3. The major reduction effect on total disomy observed after the treatment is due to the reduction of total sexual disomy (-29.3%). The major contribution to the reduction in total sexual disomy is found to be due to a reduction in the percentage of XX disomy (-35.5%) and YY disomy (-50%).

20 These results show that a treatment with recombinant FSH in men is able to reduce significantly total aneuploidy, especially diploidy and sex chromosome disomy.

Claims:

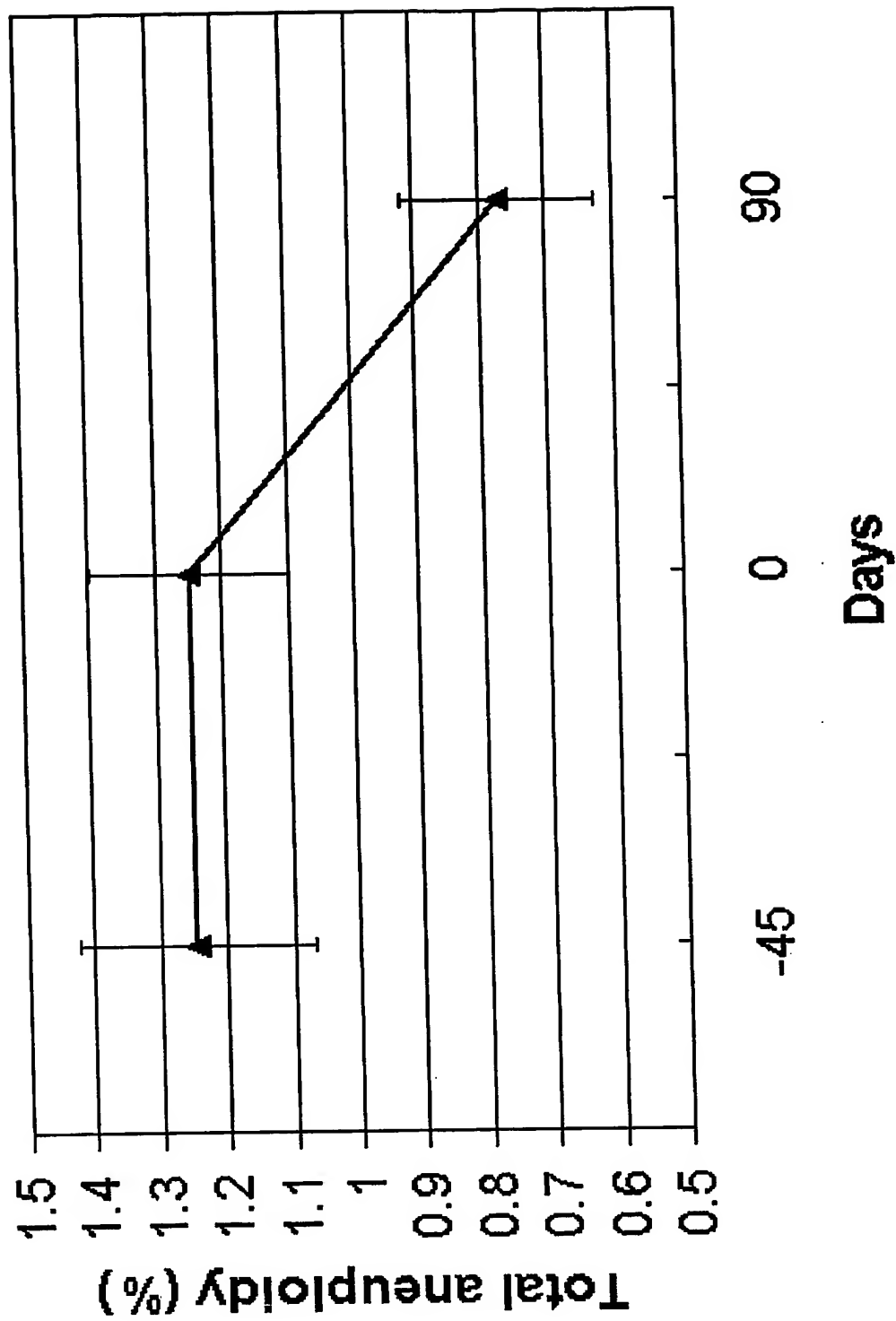
1. Use of a substance selected from Follicle Stimulating Hormone (FSH) and FSH variant for the preparation of a pharmaceutical composition for the treatment and/or the reduction of gamete chromosomal alterations in a male.
2. Use according to claim 1 wherein the gamete chromosomal alterations are numerical alterations.
3. Use according to claims 1 or 2 wherein the gamete numerical chromosomal alterations is spermatozoa diploidy.
4. Use according to any of the preceding claims wherein the gamete numerical chromosomal alterations is sexual chromosome disomy.
5. Use according to any of the preceding claims wherein the male is human.
6. Use according to any of the preceding claims wherein the substance is FSH.
7. Use according to any of the preceding claims wherein the substance is rFSH.
8. Use according to any of the preceding claims wherein the substance is administered on alternate days.
9. Use according to any of the preceding claims wherein the substance is administered at or about 75 to 300 IU/dose.
10. Use according to any of the preceding claims wherein the substance is administered at or about 150 IU/dose.
11. Use according to any of the preceding claims wherein the substance is rFSH and the human male is suffering from gamete aneuploidy.

Abstract

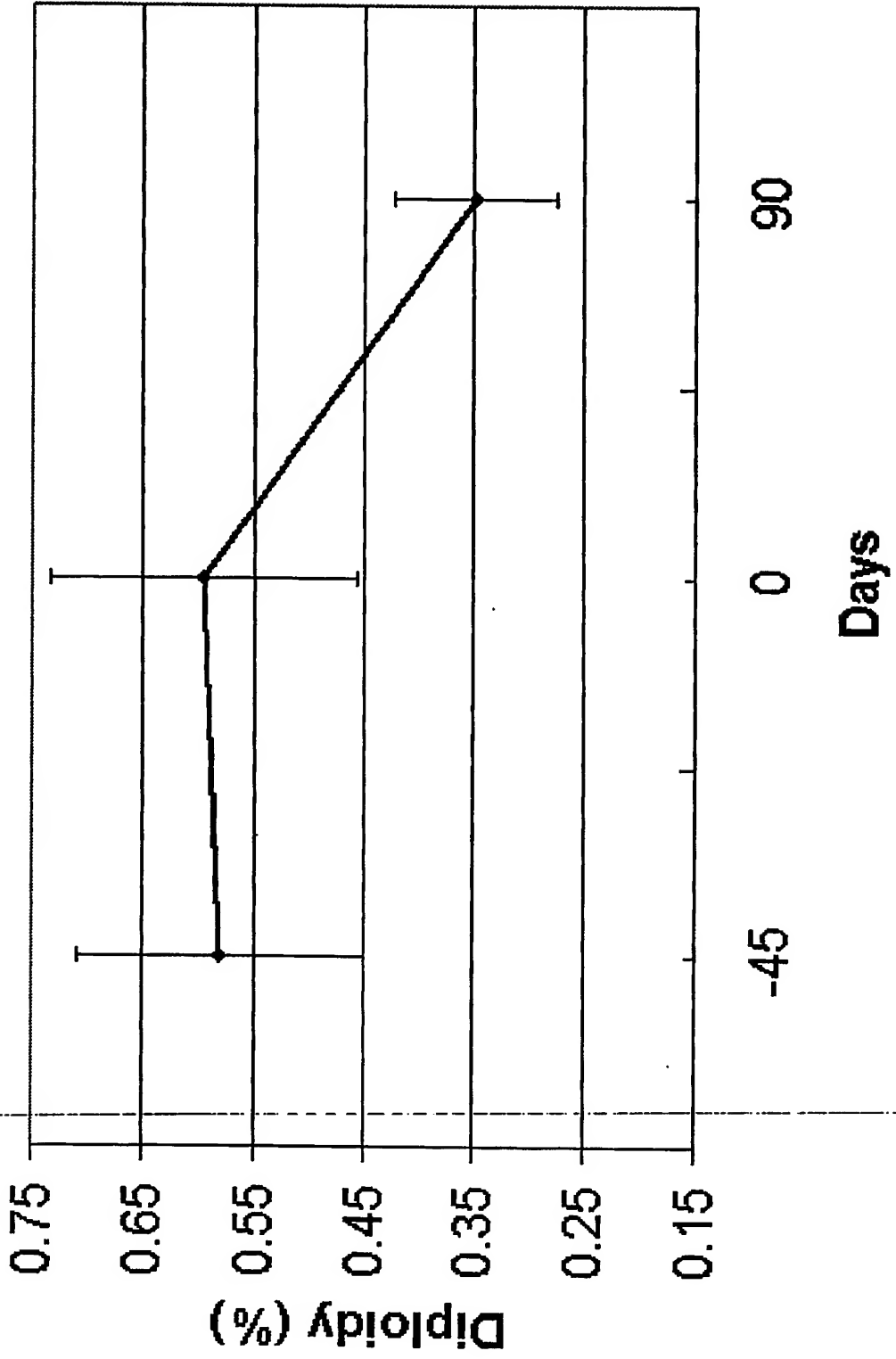
The present invention relates to the use of a substance having a FSH activity for reducing gamete chromosomal alterations in a male, more specifically in men suffering from spermatozoa aneuploidy.

1/3

Figure 1

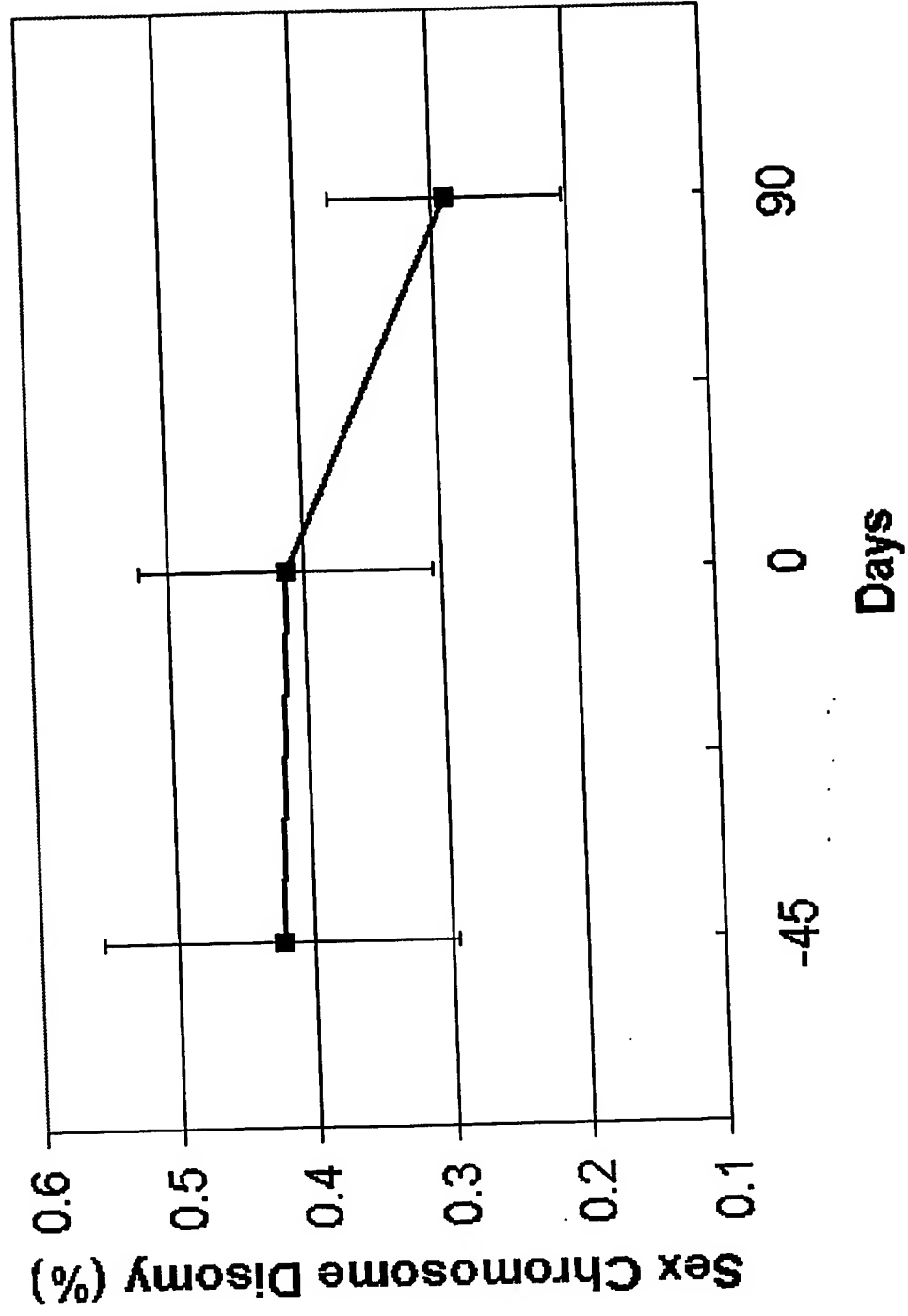


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Figure 2



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3/3
Figure 3



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